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CHARACTERISTICS OF A CA^{++}ATPASE ENZYME IN THE EPIDERMIS OF MOLTING BLUE CRABS, CALLINECTES SAPIDUS

BY

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Abstract

Epidermal Ca^{++}-activated ATPase was investigated in relation to the molt cycle of the blue crab, Callinectes sapidus. Premolt and postmolt tissue samples demonstrated significant elevations in Ca^{++}-activated ATPase activity in comparison to intermolt tissue samples. These results support the suggestion that this enzyme may participate in demineralization of the old cuticle, as well as calcification of the new cuticle.

The enzyme is localized in the heavy particulate-nuclear fraction and is distinguished from mitochondrial Ca^{++}ATPase found in other tissues by differences in sensitivity to mersalyl acid. The enzyme is most active at 49°C and has highest activity at pH values near 6.0. Reaction of the Ca^{++}ATPase with its substrate ATP produces a Vmax of 17.3 nmoles Pi mg^{-1} protein $minute^{-1}$ in intermolt crabs. The Km value for ATP is 3.61×10^{-4}M and the Q_{10} is 2.48.

INTRODUCTION

The crustacean cuticle, replaced during each molt, is a proteinaceous structure that consists of an exterior epicuticle, pigmented exocuticle, calcified endocuticle, and an uncalcified membranous layer (Warner, 1977). Chitin is a constituent of all but the epicuticle while Ca^{++}, mainly complexed with carbonate, appears in all but the innermost membranous layer. This complex integument is secreted by a single low cuboidal epithelium of closely packed cells which comprise the epidermis (Johnson, 1980).

The intermolt epidermis of Callinectes sapidus is characterized by a low cuboidal layer of cells interspersed with mature tegmental glands and pigmented melanophores. At impending molt, these epidermal cells increase in height and many new tegmental glands associated with cuticle secretion appear. Premolt subepidermal spongy connective tissue also possesses large numbers of calcium-containing reserve inclusions. Postmolt epidermis is distinguished by a diminishing number of these reserve inclusions (Johnson, 1980). The epidermal cells remain enlarged, however, probably due to the rapid influx of water at ecdysis. These cells lack the glycogen reserves found in intermolt and premolt samples because of the cessation of feeding during the actual molt (Passano, 1960).

Although the epidermis and its associated tegmental glands are thought to be responsible for the postmolt mineralization of the new cuticle, the mechanism of Ca^{++} transport is not well understood. A calcium ion activated adenosine triphosphate phosphohydrolase (Ca^{++}-ATPase) has been previously described in the Callinectes sapidus hepatopancreas and is responsible for Ca^{++} accumulation in the form of calcium phosphate during shell resorption (Becker et al., 1974; Chen et al., 1974). Ca^{++}ATPases have been documented previously in such diverse tissues as red blood cells (Wins and Schoffeniels, 1966; Wolf, 1970; Schatzmann and Rossi, 1971), skeletal muscle (Meissner et al., 1973; Ikemoto, 1975; Bennet et al., 1980), rat liver (Lehninger, 1971; Iwasa et al., 1982), teleost brain (Trams and Lautr, 1978), and rat heart tissue (Jacobus et al., 1975).

Recent analysis of resorption and deposition of Ca^{++} in the carapace of the shore crab, Carcinus maenas, has suggested several mechanisms for the transport of calcium. Ultrastructural studies during the premolt stage revealed that when epidermal cells assume a columnar shape, apical membrane surface area above the junctional level is reduced and that Ca^{++} is actively moved across the larger basolateral membrane into the hemolymph (Hopkin and Nott, 1970). This Ca^{++} is subsequently stored as reserve inclusions in the hepatopancreas. The deposition of new cuticle and subsequent increase in apical membrane during late premolt is thought to trigger the

extrusion of stored Ca^{++} (Roer, 1980).

Further studies on Ca^{++} flux across the epidermis in Carcinus maenas demonstrated active inward transport of Ca^{++} with maximal rates occurring at stage D_2 (mid premolt), while active outward transport occurs immediately postmolt with maximal rates achieved in stages A_1 and A_2 of the molt cycle. Cyanide inhibition of postmolt Ca^{++} incorporation suggested an energy dependent pathway. Ca^{++} translocation across epidermal tissue has been further linked to an ATPase through exhibition of quercitin sensitivity since quercitin has been shown to inhibit various other previously described ATPases (Roer, 1980). Towle and Mangum (1983) have noted elevation of Na^{+}-K^{+}-dependent ATPase activity in the epidermis of Callinectes sapidus during late premolt and throughout the postmolt phase of the molt cycle. To date, however, no studies have been published on epidermal Ca^{++}-dependent ATPase enzymes in the blue crab. This paper will describe a molt cycle related Ca^{++}-dependent ATPase in the epidermis of Callinectes sapidus.

MATERIALS AND METHODS

Maintenance of Specimens

Specimens of Callinectes sapidus, the blue crab, used to supply tissue for the molting stage studies were obtained in Wachapreague, Virginia and were maintained in running natural sea

water (30 °/oo) tanks. Molt cycle stages were determined according to Passano (1960) (Table I). Subcarapace epidermal tissue was dissected free of muscle tissue and placed in ice-cold homogenizing medium composed of 0.25 M sucrose, 6 mM ethylenediamine tetraacetic acid, and 20 mM imidazole (pH 6.8 with glacial acetic acid), and frozen at -20°C. Tissues were maintained in the frozen state during transport and storage at the University of Richmond until assay.

Epidermal samples for all other procedures were obtained from C_4 intermolt crabs purchased from a local seafood market. All crabs were isolated in individual compartments within an environmental chamber to prevent cannibalism. The temperature was regulated at 18°C, the photoperiod at a 12-hour light/dark cycle, and the salinity of the water at 27-28 °/oo. Sea water was prepared by diluting Instant Ocean Synthetic Sea Salts (Aquarium Systems, Inc.) with deionized tap water. The crabs were fed commercial squid twice weekly.

Preparation of Tissue for Analysis

Preparation of tissue was accomplished by dissecting 0.4 - 0.8 g of darkly pigmented epidermis from the dorsal carapace of the animal following shell removal. Ten volumes of homogenizing medium and 0.1 volume of 10% sodium deoxycholate were added to the weighed tissue

which was then homogenized in a teflon-glass homogenizer at 1,725 rpm using 18 strokes. The resulting homogenate was filtered through two thicknesses of cheesecloth. Throughout this procedure, samples were maintained on ice to restrict the activity of proteolytic enzymes.

Assay of Ca^{++} ATPase Activity

Enzymatic assay of the filtered homogenate was accomplished in a reaction mixture utilizing 0.2 ml of 200 mM imidazole (pH 7.8 with acetic acid), 0.3 ml of 100 mM $CaCl_2$, and 0.2 ml of 25 mM Tris-ATP (Sigma Chemical Corp.) in 1.2 ml distilled water. The effects of the poisons ouabain and mersalyl acid on enzyme activity were studied by substituting 0.2 ml of 0.01 M ouabain or 0.01 M mersalyl acid for H_2O. The actual assay procedure was a modification of that of Towle et al. (1976) and Fox and Rao (1978) with the following changes.

Each reaction tube was vortexed, allowed to equilibrate in a 30°C circulating water bath for five minutes, and vortexed again upon the addition of 0.1 ml homogenate. The assay mixture was incubated for 20 minutes and the reaction was terminated by the addition of 2.0 ml of cold 10% trichloroacetic acid. The tubes were placed in an ice bath for 10 minutes, then centrifuged in an International Centrifuge Model PR-2 at 2,000 rpm for 20 minutes at

$2^{o}C$. Blanks containing no enzyme and those containing enzyme but no Ca^{++} were run simultaneously.

Following centrifugation, 2.0 ml of the supernatant were removed and diluted with 2.0 ml distilled water for inorganic phosphate assay. Absorbance was measured spectrophotometrically at 660 nm with a Bausch and Lomb Spectronic 20 following the addition of 0.8 ml acid molybdate and 0.2 ml Fiske Subbarow reducer (Sigma Chemical Corp.) according to the Fiske-Subbarow method (Fiske and Subbarow, 1925). Protein was measured spectrophotometrically at 595 nm using a coomassie blue binding assay (Bradford, 1976; BioRad Laboratories) with 1 mg/ml bovine serum albumin as a standard. Variations of the Ca^{++}ATPase assay included experiments altering pH, temperature, ATP concentration, and Ca^{++} concentration.

Optimum pH was determined by assay of tissue homogenates at pH values ranging from 3.0 to 12.0. Concurrent assays containing the poison mersalyl acid were also performed. The pH values were tested for each tube to ensure the proper H^{+} as additions of mersalyl acid were shown to significantly alter pH. Aliquots of 6 M KOH were required to reestablish expected H^{+} concentration.

Optimum temperature required for enzymatic activity was assessed by conducting assays at temperatures ranging from 3^{o} to $71^{o}C$. Reaction tubes containing the poison ouabain were assayed as well. The Ca^{++} of each reaction mixture was 14 mM rather than

15 mM as in all other procedures. Although the temperature optimum was determined to be 49°C, all further experiments were conducted at 30°C due to difficulties in maintaining a nonfluctuating elevated water temperature in the presence of considerably cooler air temperatures.

ATP concentration ranging from 0 to 10 mM were used to measure the effect of substrate concentration on the Ca^{++}ATPase. The Lineweaver-Burk analysis was used in determining Km and a Michaelis-Menten plot was used to obtain the Vmax of the reaction. The effects of Ca^{++} concentration (0 to 20 mM) with and without the two poisons were also examined. All data from these kinetic studies were expressed as nmoles Pi released per mg protein per minute.

Subcellular Localization of the Ca^{++}ATPase Enzyme

After removing aliquots for total Ca^{++}ATPase assay, the remaining filtered homogenate was spun in a Beckman Model L3-50 Ultracentrifuge at 1,150 x g for 10 minutes to remove the first fraction containing nuclei and unbroken cells. The resulting supernatant was centrifuged at 11,000 x g for 35 minutes to remove the mitochondrial pellet. The microsomal fraction was separated with a spin of 105,000 x g for 60 minutes. All pellets were resuspended in homogenizing medium for assay of Ca^{++}ATPase activity.

Mechanical shear forces were required to completely resuspend the pellets. To assure the reliability of the fractionation procedure, a concurrent assay for cytochrome oxidase was performed to determine the extent of mitochondrial content. After resuspension of each pellet and collection of the soluble fraction, the previously described procedure was used for analysis of the enzyme and a mersalyl acid treated duplicate. The cytochrome oxidase assays of each fraction were monitored on a Bausch and Lomb Spectronic 700 spectrophotometer (Wharton and Tzagoloff, 1967) and absorbance units per minute were recorded on an Omniscribe recorder. Cytochrome oxidase activity was measured as absorbance mg^{-1} protein $minute^{-1}$.

Analysis of Data

Mean values for each premolt and postmolt stage were tested for statistical significance in comparison to the mean value from the C (intermolt) crabs. The statistical test utilized was the Student's t-test with significance established at the $p = .05$ probability level. Curves generated from other data were fitted visually to data points.

RESULTS

Ca^{++}ATPase activity in subcellular fractions of pooled epidermal tissue was elevated in both nuclear and mitochondrial fractions

(Figure 1). A concurrent cytochrome oxidase assay indicated minimal mitochondrial contamination in other fractions. Inhibitor studies suggested the presence of two distinct Ca^{++}ATPase enzymes. The nuclear ATPase activity was impeded by mersalyl acid whereas its mitochondrial counterpart was not.

There was a linear correlation (.9065) between Ca^{++} concentration and enzyme activity at low Ca^{++} concentrations with saturation occurring at 12 mM Ca^{++}. Mersalyl acid reduced Ca^{++} stimulation as enzyme activity peaked bewteen 6.0 and 8.0 Ca^{++} and inhibition occurred above 10 mM Ca^{++}. Ouabain produced a slight stimulation throughout the Ca^{++} concentration range. A combination of mersalyl acid and ouabain produced a maximal rate of less than 2.0 nmoles Pi mg^{-1} protein $minute^{-1}$ (Table II).

Maximal enzyme activity occurred at 5 mM ATP signifying substrate saturation with apparent Km calculated to be 3.61×10^{-4}M. Both Lineweaver-Burk and Michaelis-Menten data analysis appear in Figure 2. Vmax for intermolt epidermal samples was found to be 17.34 nmoles Pi mg^{-1} protein $minute^{-1}$.

The pH optimum was shown to be approximately 6.0 where the enzyme was three times more active than at pH 7.8, the pH of the assay medium (Figure 3). Mersalyl acid resistant ATPase had a slightly more alkaline (7.0) pH optimum.

In temperatures ranging from 3° to 71°C, ATPase activity increased to a maximum at 49°C and decreased at temperatures above 50°C suggesting enzymatic denaturation (Figure 4). Ouabain treated samples produced the characteristic slight stimulation throughout the entire temperature range with the exception of the optimum temperature for the untreated sample. The calculated Q_{10} between 20° and 30°C for the untreated sample was 2.48 whereas that of the ouabain treated sample was 2.20.

Table III shows the activity of epidermal Ca^{++}ATPase in relation to the molt cycle. Significantly higher activities were found at stages D_2, D_4, and $D_{4(late)}$ of premolt and stages A_1, A_2, and B_2 of postmolt in comparison to stage C intermolt samples. Maximal activity during premolt occurred at stage D_2 during calcium resorption. Ca^{++}ATPase activity during cuticle deposition at postmolt was significantly higher than that of both intermolt and premolt stages. Maximal Ca^{++}ATPase rate occurred during stage B_2 when the majority of the calcification of the endocuticle occurs.

DISCUSSION

This study describes a Ca^{++}-activated ATPase in the epidermis of Callinectes sapidus. Whether or not this enzymatic activity is secondary to structural alteration of the epidermis, it must be considered as a possible mechanism for the translocation of Ca^{++}

during the molt cycle.

In Austropotamobius pallipes, a freshwater crayfish, hemolymph Ca^{++} concentration was shown to decrease at premolt, followed by a rapid uptake against an electrochemical gradient at postmolt (Greenaway, 1974a; 1974b). Towle and Mangum (1983) and Guderley (1977) noted decreases in hemolymph Ca^{++} concentration at postmolt in Callinectes sapidus respectively, due to Ca^{++} incorporation into the forming cuticle. The epidermis may be important in actively transporting this Ca^{++} both during the premolt storage phase and the postmolt mineralization phase of the molt cycle.

Proposed mechanisms for Ca^{++} transport by the epidermis of molting crabs include a Na^{+}– Ca^{++} exchange (Gofraind-Debecker and Gofraind, 1980) and an energy dependent ion carrier (Roer, 1980). Unidirectional flux experiments on isolated Carcinus maenas epidermis demonstrated a ouabain- and quinine-sensitive, Na^{+} -dependent Ca^{++} movement across the epidermis, suggesting a Na^{+}– Ca^{++} exchange across the epithelium as a whole (Roer, 1980). No Ca^{++} flux was demonstrable in the absence of Na^{+}. The Ca^{++}ATPase in the epidermis of Callinectes sapidus measured in homogenate shows activity in a Na^{+} free medium. Although tissue contained endogenous Na^{+}, amounts present cannot be expected to account for the large activity increase seen at postmolt. A Na^{+}– Ca^{++} exchange in intact tissue cannot be ruled out; however, there seems to be evidence in this study for a Na^{+} -independent Ca^{++}ATPase.

The Callinectes sapidus epidermal Ca^{++}ATPase is sensitive to the sulfhydryl disrupting poison, mersalyl acid, and is insensitive to ouabain. These properties are also displayed in rat liver plasma membrane Ca^{++}ATPase (Iwasa et al., 1982), eel branchial Ca^{++}ATPase (Fenwick, 1976), and hen oviduct shell gland Ca^{++}ATPase (Coty and McConkey, 1982). Ouabain may stimulate Ca^{++}ATPase activity by eliminating competition by the Na^{+}–K^{+} -dependent ATPase for available ATP.

Mersalyl acid sensitivity indicates the importance of disulfide bonds in the preservation of the three dimensional conformation and subsequent activity of the Ca^{++}ATPase. The presence of these sulfide groups is substantiated by the exhibition of thermal stability. High energies of dissociation of S–S bonds in relation to H–H bonds may account for the ability of the Ca^{++}ATPase to function and utilize temperatures where other competitive enzymes are denatured (Ma et al., 1974; Fox and Rao, 1976; Levitsky et al., 1976; Guo and Messer, 1978; Trams and Lauter, 1978).

The pH optimum of the epidermal Ca^{++}ATPase (6.0) was remarkable in that most other Ca^{++}ATPase systems demonstrated pH optima above 7.0. In vitro studies of avian oviduct, however, have demonstrated a rapid increase of Ca^{++} translocation and incorporation into the egg shell membrane in association with a marked drop of blood pH in the distal isthmus (Laklia, 1981). Significant metabolic acidosis and associated pH depression have recently been noted in

the hemolymph of Callinectes sapidus during stages A_2 through B_2 of the molt cycle (McMahon et al., 1983). This pH shift may contribute to the marked rise of Ca^{++}ATPase activity displayed during postmolt.

Trams and Lauter (1978) described Ca^{++}ectoATPases in brain plasma membrane which are associated with increased permeability to water and ions. Data from this study show a mersalyl acid sensitive Ca^{++}ATPase in the heavy particulate-nuclear fraction. This fraction may contain apical membrane components but, as yet, no suitable method for apical membrane purification for crustacean tissues is known. If the ectoATPases can be considered analogous to apical membrane ATPases, proliferation documented in apical membrane (Green and Neff, 1972) from late premolt through postmolt would indicate elevations of enzyme laden tissue and a subsequent elevation of measurable activity as is noted in this study. In intact epidermis, Ca^{++}ATPase and Na^{+}–K^{+} ATPase may act synergistically during fluid influx at postmolt, linking Na^{+} and Ca^{++} transport. Epidermal Na^{+}– K^{+} ATPase also demonstrates significant postmolt activation in Callinectes sapidus (Towle and Mangum, 1983).

The epidermal Ca^{++}ATPase of Callinectes sapidus required a high concentration (12 mM) of Ca^{++} for maximal activity. If this enzyme is linked to Ca^{++} transport, the requirement of a high Ca^{++} concentration for enzyme activation would be advantageous in restricting the enzyme to function only during premolt and postmolt

when large quantities of Ca^{++} have to be rapidly translocated (Fox and Rao, 1978). The maintenance of intracellular Ca^{++} requires strict regulation in the micromolar range and may be attributed to mitochondrial Ca^{++}ATPases (Carafoli and Crompton, 1978). Mitochondrial Ca^{++}ATPases in Callinectes sapidus epidermis were distinguishable from the heavy particulate Ca^{++}ATPase by differences in sensitivity to mersalyl acid. Characterization studies of each fraction are indicated for future study in order to completely distinguish these two different Ca^{++} transporting enzymes.

LITERATURE CITED

Becker, G.L., C.H. Chen, J.W. Greenawalt, and A.L. Lehninger. 1974. Calcium phosphate granules in the hepatopancreas of the blue crab Callinectes sapidus. J. Cell Biol. 61, 316-326.

Bennet, J.P., K.A. McGill, and G.B. Warren. 1980. The role of lipids in the functioning of a membrane protein: The sarcoplasmic reticulum pump. Curr. Top. Mem. Transp. 14, 127-164.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Crarfoli, E. and M. Crompton. 1978. The regulation of intracellular calcium. Curr. Top. Mem. Transp. 10, 151-216.

Chen, C.H., J.W. Greenawalt, and A.L. Lehninger. 1974. Biochemical and ultrastructural aspects of Ca^{++} transport by mitochondria of the hepatopancreas of the blue crab, Callinectes sapidus. J. Cell. Biol. 61, 301-315.

Coty, W. A. and C.L. McConkey, Jr. 1982. A high affinity calcium stimulated ATPase activity in the hen oviduct shell gland. Arch. Biochem. Biophys. 219, 444-453.

Fenwick, J. 1976. Effect of stanniectomy on calcium activated adenosinetriphosphatase activity in the gills of freshwater adapted North American eels, Anguilla rostrata Lesueur. Gen. Comp. Endocrinol. 29, 383-387.

Fiske, C.F. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Fox, F.R. and K.R. Rao. 1978. Characteristics of a Ca^{2+}-activated ATPase from the hepatopancreas of the blue crab, Callinectes sapidus. Comp. Biochem. Physiol. 59B, 327-331.

Gofraind-Debecker, A. and T. Gofraind. 1980. Calcium transport system: A comparative study in different cells. Int. Rev. Cytol. 67, 141-170.

Green, J.P. and M.R. Neff. 1972. Survey of the fine structure of the fiddler crab. Tissue and Cell 4, 137-171.

Greenaway, P. 1974a. Calcium balance at the premoult stage of the freshwater crayfish Austropotamobius pallipes (Lereboullet). J. Exp. Biol. 61, 27-34.

_____, 1974b. Calcium balance at the postmolt stage of the freshwater crayfish Austropotamobius pallipes (Lereboullet). J. Exp. Biol. 61, 35-45.

Guderley, H. 1977. Muscle and hypodermal ion concentrations in Cancer magister: Changes with the molt cycle. Comp. Biochem. Physiol. 56A, 155-159.

Guo, M.K. and H.H. Messer. 1978. A comparison of Ca^{2+}-, Mg^{2+}-ATPase and alkaline phosphatase activities of rat incisor pulp. Calcif. Tiss. Res. 26, 33-38.

Hopkin, S.P. and J.A. Nott. 1979. Some observations on concentrically-structured, intracellular granules in the hepatopancreas of the shore crab, Carcinus maenas. J. Mar. Biol. Assoc. U. K. 59, 867-877.

Ikemoto, N. 1975. Transport and inhibitory Ca^{2+}-binding sites on the ATPase enzyme isolated from sarcoplasmic reticulum. J. Biol. Chem. 250, 7219-7224.

Iwasa, Y., T. Iwasa, K. Higashi, K. Matsui, and E. Miyamoto. 1982. Demonstration of a high affinity Ca^{2+}-ATPase in rat liver plasma membranes. Biochem. Biophys. Res. Comm. 105, 488-494.

Jacobus, W.E. R. Tiozzo, G. Lugli, A.L. Lehninger, and E. Carafoli. 1975. Aspects of energy linked Ca^{2+} accumulation by rat heart mitochondria. J. Biol. Chem. 250, 7863-7870.

Johnson, P.T. 1980. Histology of the Blue Crab, Callinectes sapidus. pp. 64-83. New York: Praeger Publishers.

Laklia, J. 1981. Ca-transport in the distal segments of the avian oviduct: An in vitro study. Acta Vet. Acad. Hung. 29, 283-292.

Lehninger, A.L. 1971. A soluble, heat labile, high affinity Ca^{2+} binding factor extracted from rat liver mitochondria. Biochem. Biophys. Res. Commun. 42, 312.

Levitsky, D. O., M.K. Aliev, A.V. Kuzmin, T.S. Levchenko, V. N. Smirnov, and E.I. Chazov. ±976. Isolation of calcium pump system and purification of calcium ion-dependent ATPase from heart muscle. Biochim. Biophys. Acta 443, 468-484.

Ma, S.W.Y., Y. Shami, H.H. Messer and D.H. Copp 1974. Properties of a Ca^{2+}-ATPase from the gill of rainbow trout (Salmo gairdneri). Biochim. Biophys. Acta 345, 243-251.

McMahon, B.R., P.L. deFur, C.P. Mangum, and M.G. Wheatley. 1983. Compensation for changes in acid base status during the molting cycle in the blue crab Callinectes sapidus. Amer. Soc. Zool. 23, 952 (abstract).

Meissner, G. ±973. ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum. Biochim. Biophys. Acta 389, 51-68.

Passano, L.M. 1960. Molting and its control. In Physiology of the Crustacea (edited by Waterman, T.H.) vol. 1., 473-536. New York: Academic Press.

Roer, R.D. 1980. Mechanisms of resorption and deposition of calcium in the carapace of the crab Carcinus maenas. J. Exp. Biol. 88, 205-218.

Schatzmann, H.J. and G.L. Rossi. 1971. Ca^{++} and Mg^{++} activated membrane ATPases in human red cells and their possible relation to cation transport. Biochim. Biophys. Acta 241, 379-392.

Trams, E.G. and C.J. Lauter. 1978. A comparative study of brain Ca^{2+}-ATPases. Comp. Biochem. Physiol. 59B, 191-194.

Towle, D.W., G.E. Palmer, and J.L. Harris III. 1976. Role of gill Na^{+} +K^{+} -dependent ATPase in acclimation of blue crabs (Callinectes sapidus) to low salinity. J. Exp. Biol. 196, 315-322.

_____, and C.P. Mangum. 1983. Ionic regulation and transport ATPase activities during the molt cycle in the blue crab, Callinectes sapidus. J. Crustacean Biol. (in press).

Warner, G.F. 1977. The Biology of Crabs. pp 1-7. London: Van Nostrand Reinhold Company.

Wharton, D.C. and A. Tzagoloff. 1967. Cytochrome oxidase from beef heart mitochondria. In Methods of Enzymology (edited by Estabrook, R.W. and M.E. Pullman) Vol. 10, 245-250.

Wins, T. and E. Schoffeniels. 1966. Studies on red cell ghosts ATPase system: Properties of a Mg^{++} + Ca^{++} dependent ATPase. Biochim. Biophys. Acta 120, 341-350.

Wolf, H.V. 1970. Purification of the Ca^{2+}-dependent ATPase of human erythrocyte membranes. Biochim. Biophys. Acta 266, 361-375.

Table I. Brachyuran intermolt stages used in staging Callinectes sapidus during collection of molt cycle samples (Passano, 1960).

Stage	Name	Characteristics	Activity Level	Feeding	$\%H_2O$
Stage A	Newly				
A_1	Molted	Continued water adsorption and initial mineralization	Slight	None	---
A_2	Soft	Exocuticle mineralization	Some	None	86
Stage B	Paper Shell				
B_1		Endocuticle secretion begins	Considerable	None	85
B_2		Active endocuticle formation, chelae hard; tissue growth begins	Full	Starts	83
Stage C	Hard				
C_1		Main tissue growth	Full	Yes	80
C_2		Tissue growth continues	Full	Yes	76
C_3		Completion of exoskeleton; membraneous layer formed	Full	Yes	68
C_4		Intermolt; major accumulation of food reserves	Full	Yes	61
Stage D	Proecdysis				
D_0		Epidermal and hepatopancreatic activation	Full	Yes	60
D_1		Epicuticle formed	Full	Yes	---
D_2	Peeler	Exocuticle secretion	Full	Reduced	---
D_3		Major portion of skeletal resorption	Reduced	None	---
D_4	About to Molt	Ecdysial sutures open	Slight	None	Rise
Stage E	Molt	Rapid water uptake and exuviation	None	None	Rapid Rise

on Ca^{++} concentration*.

+Ouabain		
3.50 ± 0.39	(4)	
3.25 ± 0.00	(1)	
8.64 ± 0.42	(5)	
8.85 ± 0.00	(2)	
10.03 ± 0.41	(5)	
10.80 ± 0.00	(2)	
11.62 ± 1.47	(5)	
9.93 ± 0.10	(2)	
12.52 ± 2.29	(5)	
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14.44 ± 0.17	(3)	

Table II (continued).

mM Ca^{++}	SPECIFIC ACTIVITY nmoles Pi mg^{-1} protein $minute^{-1}$					
	No Inhibitors		+Mersalyl Acid		+Ouabain	
6.0	11.22 ± 0.08	(4)	5.85 ± 0.32	(5)	15.16 ± 0.34	(3)
7.0	11.70 ± 0.33	(6)	5.61 ± 0.12	(5)	14.30 ± 2.41	(4)
8.0	12.16 ± 0.72	(5)	5.67 ± 0.06	(5)	13.44 ± 2.85	(5)
9.0	13.02 ± 0.23	(4)	5.42 ± 0.11	(3)	17.32 ± 0.11	(3)
10.0	13.13 ± 0.00	(3)	5.40 ± 0.13	(2)	15.05 ± 0.39	(2)
12.0	14.74 ± 0.48	(3)	3.43 ± 0.08	(3)	----------	
14.0	14.32 ± 0.19	(4)	3.34 ± 0.24	(3)	----------	
16.0	14.32 ± 0.19	(4)	2.91 ± 0.21	(3)	----------	
18.0	14.39 ± 0.26	(4)	3.32 ± 0.00	(1)	----------	
20.0	14.51 ± 0.22	(7)	2.95 ± 0.06	(3)		

*Data expressed as mean ± standard deviation.
Sample sizes are in parentheses.

Table III. Specific activity of the epidermal Ca^{++}ATPase in molting crabs, Callinectes sapidus.

Stage	Mean**	SD	SE	N	t	P
A_1	80.97	21.63	21.63	2	2.79	.050*
A_2	150.14	30.00	17.32	4	7.15	.001*
B_1	56.08	37.88	18.94	5	1.90	.100
B_2	183.94	68.54	48.47	3	3.39	.025*
C	18.02	6.42	3.71	4	----	----
D_1	30.24	16.34	9.43	4	1.07	.400
D_2	50.86	3.19	3.19	2	4.58	.010*
D_3	31.20	0.24	0.24	2	2.05	.100
D_4	35.16	7.52	3.76	5	2.30	.050*
$D_{4(late)}$	55.56	9.80	6.93	3	3.97	.010*

*Statistically significant at the $p \leq .05$ confidence level.

** Mean expressed in nmoles Pi mg^{-1} protein $minute^{-1}$.

Figure 1. Specific activity of intermolt epidermal cell fractions in Callinectes sapidus. (A) Activity of uninhibited Ca^{++}ATPase enzyme (B) Activity of mersalyl acid treated Ca^{++}ATPase enzyme (C) Activity of the Cytochrome oxidase enzyme.

CYTOCHROME OXIDASE ACTIVITY

Δabsorbance/mg protein/minute

FRACTION

Ca^{++}ATPase Activity

nmoles Pi/mg protein/min

Figure 2. Dependence of Ca^{++}ATPase on substrate (ATP) concentration in intermolt crabs, Callinectes sapidus. Michaelis-Menten graph demonstrates Vmax occurring at substrate saturation. (Inset) Lineweaver-Burk plot used in the determination of Ca^{++}ATPase affinity for ATP.

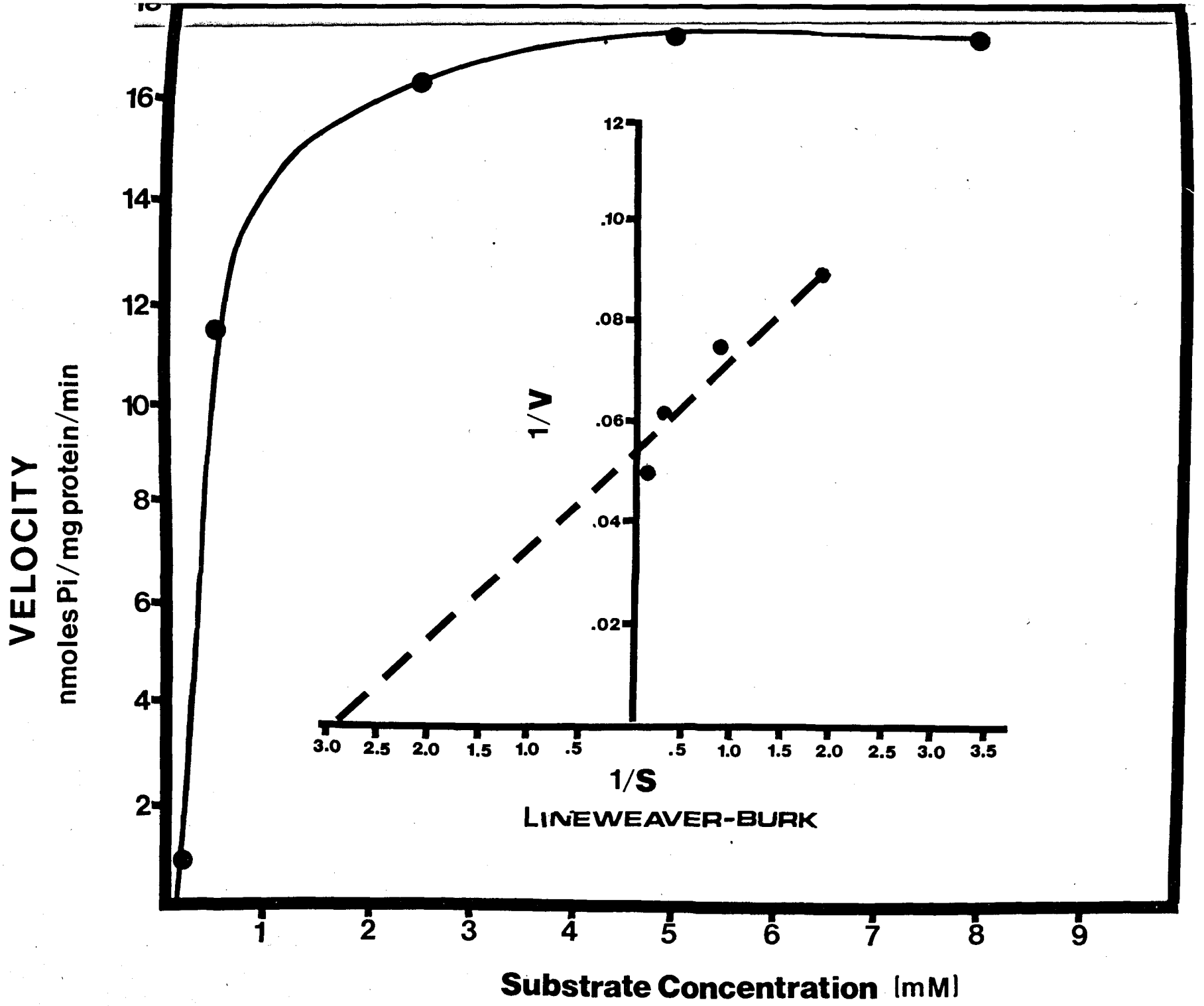

VELOCITY
nmoles Pi / mg protein/min
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
Substrate Concentration (mM)
12
.10
.08
.06
.04
.02
1/V
3.0
2.5
2.0
1.5
1.0
.5
.5
1.0
1.5
2.0
2.5
3.0
3.5
1/S
LINEWEAVER-BURK

Figure 3. Effect of pH on C_4 epidermal samples in Callinectes sapidus. Activity of uninhibited Ca^{++}ATPase (●) and mersalyl acid treated Ca^{++}ATPase (■) trials. Data represent mean ± standard deviation.

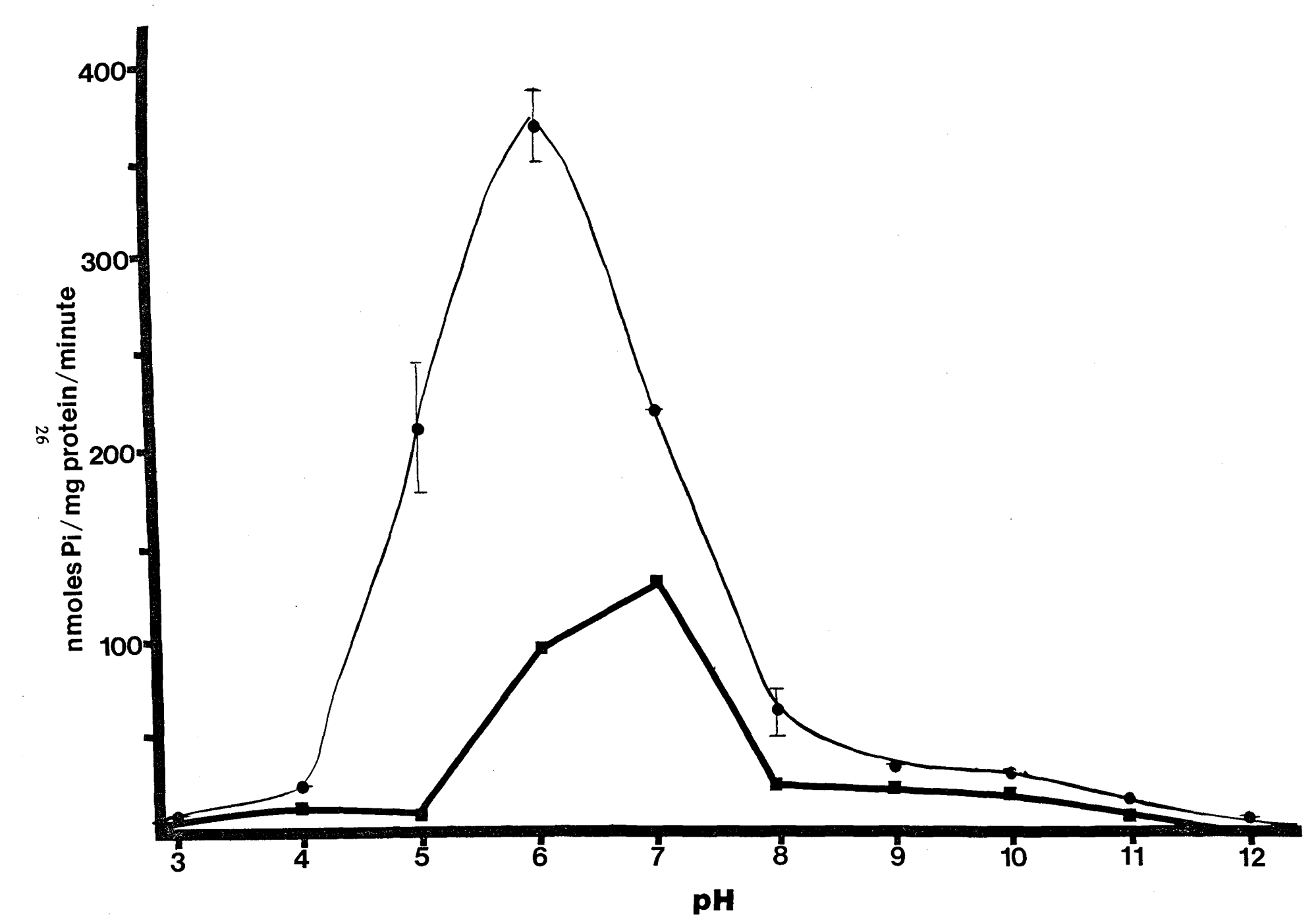
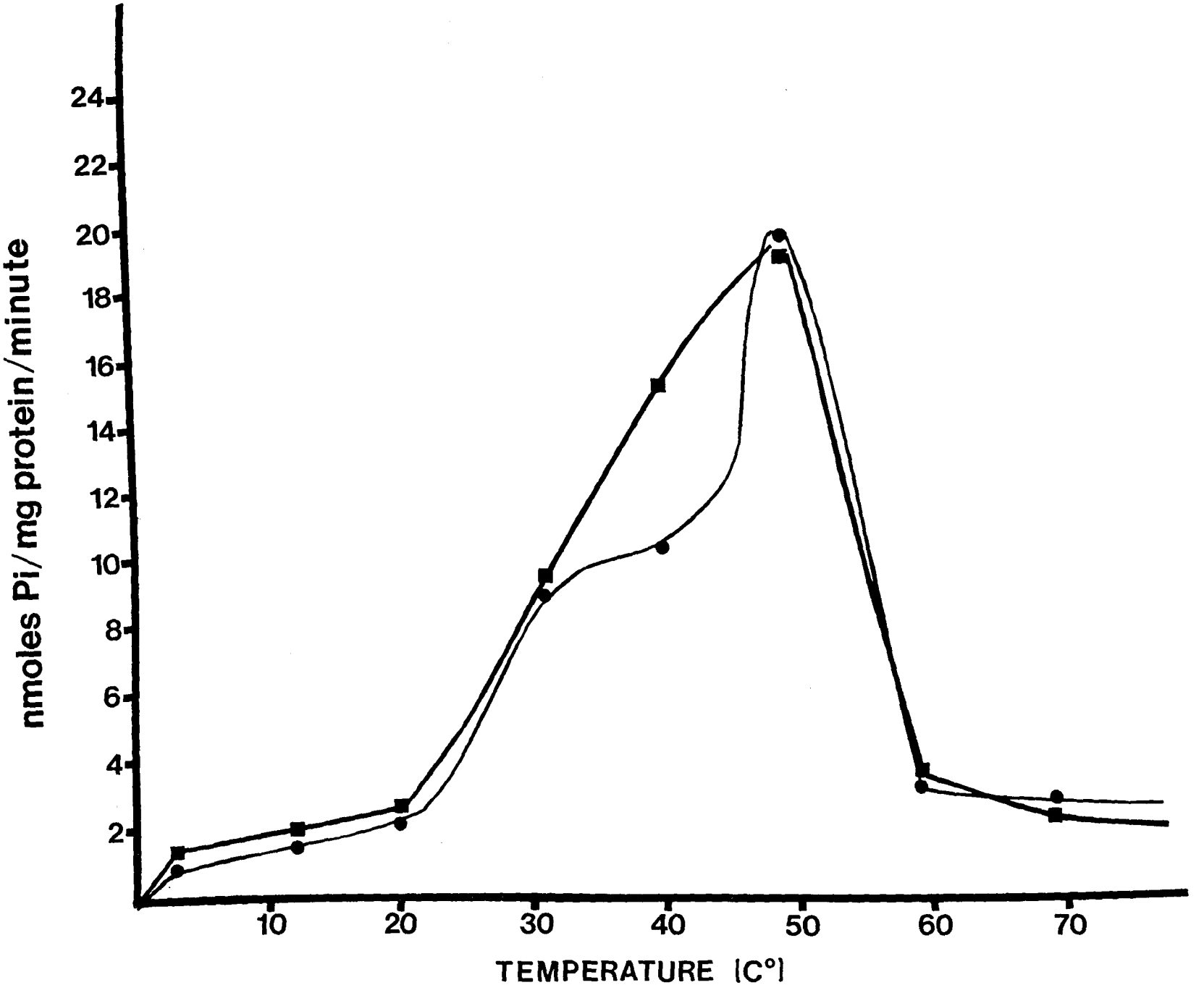

400
300
200
100
nmoles Pi / mg protein / minute
3
4
5
6
7
8
9
10
11
12
pH

Figure 4. Temperature dependence of uninhibited Ca^{++}ATPase (●) in relation to ouabain treated Ca^{++}ATPase (■) in *Callinectes sapidus*.


nmoles Pi/mg protein/minute
24
22
20
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
70
TEMPERATURE (C°)

VITA

Joanne Elizabeth Lapetina was born on December 3, 1959, in Norfolk, Virginia. She graduated with high honors from Norfolk Christian High School in June of 1977. In May, 1981, she was graduated from the University of Richmond with a Bachelor of Science degree in Biology. She completed the requirements for a Master of Science degree in Biology from the University of Richmond in 1984. She held membership in the Beta Beta Beta biological honor society and is currently affiliated with the Medical College of Virginia studying the enzymatic properties of abnormal lymphocytes associated with pediatric leukemia.